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Background—Primary pulmonary arterial hypertension (PAH) is a potentially devastating condition resulting from occlusion of the pulmonary arterioles by the formation of vascular lesions. Heterozygous mutations in the gene encoding the bone morphogenetic protein receptor type II (*BMPR2*) have been identified in both familial (FPAH) and idiopathic PAH. Mutant alleles are typically of low penetrance, indicating that other factors are required for the onset of PAH. Previous reports have suggested that the characteristic plexiform lesions in affected lungs are akin to neoplasia, showing monoclonal expansion and microsatellite instability. We hypothesized that in patients with germline mutations, *BMPR2* might behave as a classic tumor suppressor gene, with somatic loss of the wild-type allele contributing to disease progression.

Methods and Results—To test this hypothesis, plexiform and concentric vascular lesions were serially microdissected from lung explant tissue derived from 7 FPAH cases. DNA was analyzed for loss of heterozygosity at *BMPR2* and for microsatellite instability (MSI) at 5 loci. MSI was detected in 1 of 37 lesions at a single locus, BAT-26, whereas heterozygosity at *BMPR2* was retained at all informative loci. We also describe a FPAH patient carrying biallelic constitutional missense mutations of *BMPR2* who manifested disease at a stage and manner similar to heterozygous patients.

Conclusions—Taken together, these data demonstrate that MSI is uncommon in FPAH and suggest that somatic loss of the remaining wild-type *BMPR2* allele in heterozygous mutation carriers likely does not play a significant role in modulating the onset or progression of FPAH. **(*Circulation*. 2005;111:607-613.)**

Key Words: hypertension, pulmonary ■ genetics ■ lung ■ genes ■ pathology

Primary pulmonary arterial hypertension (PAH) is an often fatal condition typified by the sustained elevation of mean pulmonary artery pressure, which leads to hypertrophy and subsequent failure of the right heart. Histologically, the disease is characterized by obstruction of the small pulmonary arteries resulting from the development of vascular plexiform and concentric lesions that in turn are defined by proliferating and migrating endothelial and smooth muscle cells.[1,2] Although the majority of PAH patients spontaneously present with disease and are regarded as idiopathic cases (IPAH), up to 10% may have a family history of the condition (FPAH). Inheritance of PAH is autosomal-dominant with a sex bias to the reduced penetrance of mutant alleles.[3] We and others have demonstrated that diverse germline heterozygous mutations in the gene encoding the bone morphogenetic protein receptor type II (*BMPR2*) underlie the majority of patients with FPAH and additionally, up to 40% of apparently sporadically affected subjects.[4–7]

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BMPR-II is a type 2 receptor of the transforming growth factor (TGF)-β family of cytokines, members of which are regarded as essential for the cellular processes of proliferation, differentiation, and apoptosis. The receptor comprises 4 functional domains (ligand binding, kinase, transmembrane, and cytoplasmic) and relays intracellular signals in complex with a type I receptor (BMPR-1A/1B) via the phosphorylation of a series of cytoplasmic mediators, of which the Smad proteins are the most extensively characterized. Activated Smads, in concert with other signaling partners, translocate to the nucleus, where they regulate the transcription of target genes.[8] Although the Smad signaling pathway is regarded as

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a major target of TGF-β signal transduction, several Smad-independent substrates are now being elucidated, including the mitogen-activated protein kinases (MAPKs), extracellular signal–regulated kinase, c-Jun NH_2-terminal kinase, and $p38^{MAPK}$.[9]

Approximately 60% of the pathogenic mutations reported to date introduce premature truncation codons to the *BMPR2* transcript, most of which are anticipated to undergo nonsense-mediated decay, supporting haploinsufficiency of the protein as the molecular mechanism of disease.[10] By contrast, the remainder are point mutations that occur at functionally critical amino acid residues and have been independently demonstrated to have heterogeneous effects on important receptor functions, including ligand binding and phosphorylation of substrate.[11,12] In PAH kindreds, mutant *BMPR2* alleles display reduced penetrance, indicating that heterozygous mutation of the gene is required but not sufficient for the precipitation of disease.[2] Other genetic or environmental factors are therefore also implicated.

Two studies[13,14] observed that a proportion of abnormally proliferating endothelial cells from plexiform lesions in a series of patients with IPAH and others who had developed the disease secondary to the ingestion of appetite suppressant drugs displayed monoclonal expansion. The monoclonal cell growth within such lesions was interpreted as being akin to neoplasia, suggesting that PAH onset necessitated somatic mutations in genes that conferred a selective growth advantage to cells.[13,15] In support of this hypothesis, microsatellite instability (MSI) and/or mutation of *TGF*βRII or the proapoptotic gene *BAX* was subsequently demonstrated in a proportion of plexiform lesions from PAH lungs.[15]

Because several other genes in the TGF-β/BMP superfamily have known roles in neoplasia,[16] we hypothesized that FPAH and *BMPR2* mutation–positive IPAH might follow the classic "2-hit" model of tumorigenesis and that inactivation of the remaining wild-type *BMPR2* allele might be one of the somatic mutations necessary to precipitate disease. To test this theory, we examined microdissected pulmonary vascular concentric and plexiform lesions from 7 FPAH cases for evidence of somatic loss of the wild-type *BMPR2* allele. We also describe a PAH patient who showed a typical disease course, despite harboring constitutional missense mutations of both *BMPR2* alleles.

Methods

Subjects

Patient ascertainment was performed as previously described.[10] In brief, subjects were defined as affected if pulmonary artery mean pressure exceeded 25 mm Hg and pulmonary artery wedge pressure was normal or if postmortem analysis identified plexogenic pulmonary arteriopathy.[2] Subjects with FPAH were defined by having at least 1 affected relative displaying evidence of linkage to the *BMPR2* locus. All patients were of western European descent. Patient 1 (GER02) was of German origin with 1 affected sibling. Patient 2 (FRA01), of French origin, had an affected parent and 3 affected siblings. Patients 3 (UK01), 4 (UK07), 5 (UK11), 6 (UK13), and 7 (UK14) were from the United Kingdom and had 8, 1, 2, 1, and 1 affected relatives, respectively. Germline mutations were detected among patients and carriers of the *BMPR2* disease haplotype in families of UK01, UK11, and UK13, as previously described (Table 1).

TABLE 1. Details of FPAH Cases Comprising the Microdissection Panel

Sample	Mutation	No. of Lesions Dissected	
GER02	No mutation identified	Plexiform	3
		Concentric	2
FRA01	No mutation identified	Plexiform	5
		Concentric	4
UK01	5′ deletion (Reference 10)	Plexiform	3
		Concentric	2
UK07	No mutation identified	Plexiform	2
		Concentric	2
UK11	1806 frameshift+10 aa (Reference 10)	Plexiform	2
		Concentric	2
UK13	355 delA+8 aa (Reference 4)	Plexiform	1
		Concentric	1
UK14	No mutation identified	Plexiform	5
		Concentric	3

del indicates deletion; aa, amino acids. All other abbreviations are as defined in text.

Patient 5559, who died at the age of 20 years, was diagnosed with PAH after cardiac catheterization revealed pulmonary artery pressure of 154/69 mm Hg. This individual had a recorded paternal family history of PAH but no known maternal history. Samples were not available from either parent.

Mutation Analysis of the *BMPR2* Gene in Case 5559

DNA was isolated from peripheral blood and fresh-frozen lung samples by standard methods. The 13 coding exons of the gene and flanking intronic sequences were amplified by polymerase chain reaction (PCR) with primer pairs described elsewhere.[10] Both strands of the purified PCR product were sequenced with the same primers on an ABI377 DNA analyzer with the BigDye terminator kit. The presence of identified mutations was excluded from a panel of 150 normal chromosomes, also by direct sequencing. To determine the relative proportions of the 2 mutations in the compound heterozygote patient, PCR products generated from blood and lung sources were cloned into the PCR2.1 vector according to the manufacturer's instructions (Promega). Transformation into the *Escherichia coli* strain DH5α was performed by the heat-shock procedure and overnight cultures miniprepped with the Qiagen kit. A total of 70 colonies were prepared from each source, and sequencing was carried out as described earlier.

Laser Capture Microdissection

Serial paraffin sections (10 μm) were mounted on membrane-based slides for microdissection in conjunction with 1M1000 digital imaging software (Leica Microsystems). Immediately before microdissection, sections were stained with hematoxylin and eosin to permit identification of tissue components. At least 6 serial sections were obtained from each patient sample, and all identified vascular lesions were collected. Concentric and plexiform lesions were sampled, together with random fields of normal lung parenchyma, as detailed in Table 1. Lesions were highlighted and dissected from tissue sections with a laser beam (0.6 μm, at 32 mW, 30 Hz) and an 0.8-second pulse, as illustrated in Figure 1.

Dissected material was contained in a sterile PCR plastic collecting tube cap containing 40 μL of lysis buffer (TE [10 mmol/L Tris-HCl, 1 mmol/L EDTA], pH 8.0); 1 mg/mL proteinase K; 1% Tween-20). For DNA extraction, samples were incubated overnight at 37°C. An additional 4 μL of proteinase K (20 mg/mL) was added

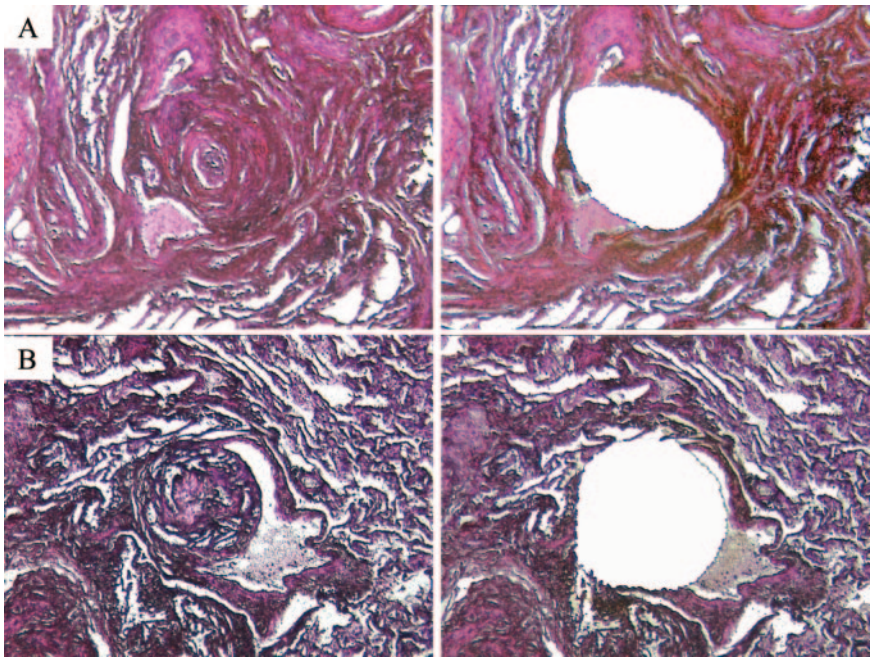

Figure 1. Laser capture microdissection. Representative lung sections before and after microdissection of (A) concentric and (B) plexiform lesions.

to each sample before each of 2 additional overnight incubations at 65°C. The samples were finally incubated at 95°C for 10 minutes to inactivate the enzyme.

MSI and Loss of Heterozygosity Analysis

MSI analysis was performed with 5 microsatellite markers: BAT-25, a thymine repeat in intron 16 of the *c-kit* oncogene; BAT-26, an adenine repeat located within the fifth intron of the DNA mismatch repair gene *hMSH2*; a guanine repeat in exon 3 of *BAX*; a 10-base adenine repeat in exon 3 of the *TGFβRII* gene[17–20]; and a 7-base adenine repeat in *BMPR2*. All familial and sporadic cases were analyzed. Primer details for these microsatellites are available on request.

Three polymorphic markers were used to detect loss of heterozygosity (LOH) at the *BMPR2* locus: a previously undescribed marker within the first intron of *BMPR2*; D2S3009, located in intron 12; and D2S2289, which maps 420 kb distal of the gene. Markers used in the MSI study were amplified with fluorescently labeled primers as either a duplex (*TGFβRII* and *BMPR2*) or a multiplex (BAT-25, BAT-26, and *BAX*) PCR. Markers used in the analysis of LOH were amplified individually. Each reaction comprised 1× Qiagen multiplex Mastermix, 0.2 μmol/L of each primer, and 2 μL of extracted genomic DNA to a final volume of 10 μL. Markers were visualized on 6% polyacrylamide gels on an ABI 377 DNA analyzer and with the use of GeneScan 3.0 software. PCR cycling conditions were as follows: preincubation at 95°C for 15 minutes to activate the polymerase; then 95°C for 30 seconds, 57°C for 90 seconds, and 72°C for 60 seconds for 40 cycles; followed by a final step of 60°C for 30 minutes.

Results

Molecular Genetic Analysis of a Patient With Biallelic *BMPR2* Mutations

Both R491Q and K512T mutations were observed as heterozygous mutations in exon 11 of *BMPR2* on sequencing of DNA isolated from 2 independent sources of tissue, namely, blood and lung. The PCR products of the exon 11 generated from these tissues were next cloned to determine (1) whether the mutations were in the *cis* or *trans* and (2) whether there was evidence of a wild-type allele, which would indicate mosaicism. Among the 140 clones analyzed, 70 from each tissue, the 2 mutations were always detected on different chromosomes, thus confirming the patient to be a compound heterozygote for *BMPR2* (Figure 2). Additionally, the mutations were present in approximately equal proportions in both tissues (Figure 2A) and importantly, there was no evidence of a wild-type allele. Neither mutation was present in the control panel of 150 normal chromosomes. Furthermore, both wild-type amino acids at positions 491 and 512 in the BMPR-II cDNA demonstrated evolutionary conservation across species, indicating their relevance to receptor function (Figure 2B).

Investigation of LOH at the *BMPR2* Locus in Microdissected Human Lung

A total of 37 vascular lesions (21 plexiform, 16 concentric) were analyzed from 7 FPAH cases, and the results were compared with normal lung parenchyma in each case. D2S3009 was informative in all 7 cases, whereas D2S2289 and the intron 1 polymorphisms were each informative in 6 cases (Table 2). Successful PCR amplification was achieved in 29 of 32 (91%) of informative lesions for intron 1, 35 of 37 (95%) for D2S3009, and 30 of 32 (94%) for D2S2289. In all cases wherein genotyping was successful, heterozygosity was retained at all informative loci (Table 2 and Figure 3).

No Evidence of MSI of 5 Genes in PAH Human Lung

MSI analysis was conducted on the same 37 lesions, together with normal parenchyma. Success rates for PCR amplification ranged from 31 of 37 lesions (84%) for *TGFBR2* to 36 of 37 (97%) for BAT-25 and *BAX* (Table 3). Aberrantly sized fragments, indicative of microsatellite mutation, were identified in a single concentric lesion from a familial case for marker BAT-26 and subsequently confirmed in a second independent PCR (Figure 4). All other results were normal (Table 3 and Figure 4).

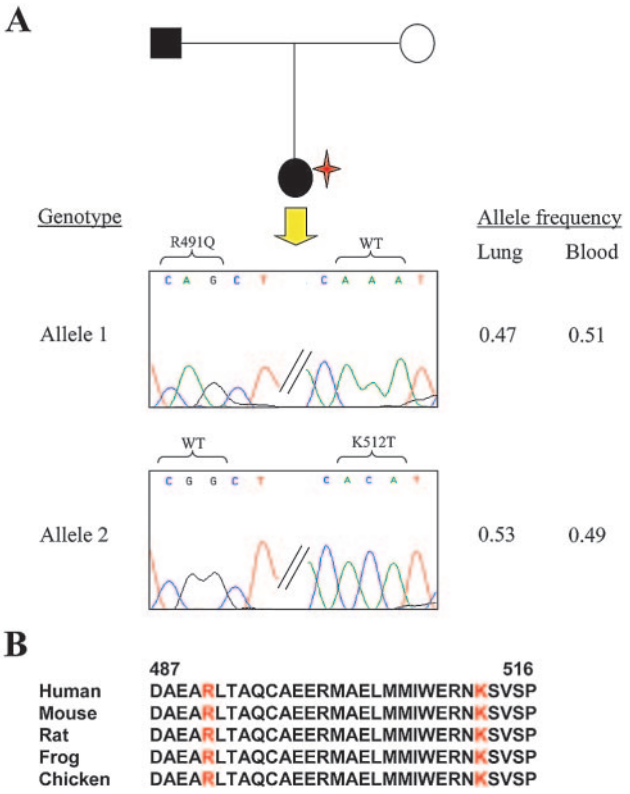

Figure 2. Mutation analysis for patient 5559. A, Pedigree represents affected individuals by filled symbols; patient 5559 is indicated by star. Segments of contiguous-sequence chromatograms are shown, demonstrating 2 different heterozygous alleles identified in this subject. Double diagonal line represents intervening 60 bases, omitted for clarity. Allele 1 has G→A transition at codon 491 and is wild type at codon 512, whereas allele 2 is wild type at codon 491 and shows A→C transversion at codon 512. Allele frequencies were obtained by sequencing 70 clones from each of lung- and blood-derived DNA. B, BMPR-II sequence from amino acids 487 to 516 demonstrating conservation of residues 491 and 512, shown in red, across species.

Discussion

Profound MSI is a hallmark of hereditary nonpolyposis colon carcinoma (HNPCC) and is also found in a proportion of sporadic HNPCC-spectrum tumors, such as endometrial carcinoma.[21] The underlying cause of MSI is a defect in mismatch repair, which results in tumorigenesis through an accumulation of somatic mutations in genes important for regulating cell cycle, growth, or apoptosis. A lower level of MSI occurs in tumors that are outside the HNPCC spectrum. Previous studies of endothelial cells microdissected from plexiform lesions of PAH lungs have shown monoclonal expansion in 17 of 22 lesions (77%) from 4 patients and microsatellite mutation rates ranging from 21% for *BAX* to 50% for *BAT26*.[13,15] This suggested that endothelial cell expansion in plexiform lesions is akin to neoplasia and might result from an accumulation of somatic mutations, either through MSI or other mutational mechanisms. We have now conducted similar analyses in a series of FPAH cases in whom *BMPR2* has been fully characterized. We hypothesized that at least in those cases with a germline *BMPR2* mutation, *BMPR2* might behave as a classic tumor suppressor gene and undergo somatic loss of the wild-type allele in pulmonary vascular lesions, as documented for the type I receptor *BMPR1A* in juvenile polyposis and Cowden syndromes.[22]

TABLE 2. Summary of Results From LOH Analysis

Sample	Intron 1	D2S3009	D2S2289
GER02	P=0/3	P=0/3	Uninformative
	C=0/2	C=0/1	
FRA01	P=0/5	P=0/5	P=0/5
	C=0/4	C=0/4	C=0/2
UK01	Uninformative	P=0/3	P=0/3
		C=0/2	C=0/2
UK07	P=0/2	P=0/2	P=0/2
	C=0/2	C=0/2	C=0/2
UK11	P=0/2	P=0/2	P=0/2
	C=0/2	C=0/2	C=0/2
UK13	P=0/1	P=0/1	P=0/1
	C=0/1	C=0/1	C=0/1
UK14	P=0/4	P=0/4	P=0/5
	C=0/1	C=0/3	C=0/3

P indicates plexiform; C, concentric. All other abbreviations are as defined in text. Results are expressed as number of samples displaying LOH of the total number that were successfully amplified.

We performed serial microdissection of plexiform and concentric vascular lesions and normal lung parenchyma on paraffin-embedded lung from 7 FPAH patients. All had previously been analyzed for a germline *BMPR2* mutation by a combination of direct sequencing, Southern blotting, and dosage PCR, and mutations had been detected in 3 of these cases.

Mononucleotide-repeat tracts in 5 genes were examined for MSI, including BAT-25 and BAT-26, which are the recommended diagnostic markers for HNPCC.[23] Only 1 mutation was found, in a single gene (BAT-26) in 1 concentric lesion; no mutations were identified among 19 plexiform lesions, in contrast with the results of Yeager et al.[15] There are 2 significant differences between the 2 studies that might account for this. First, Yeager et al[15] used whole-genome amplification (WGA) of microdissected DNA before analysis of individual genes. In the present study, an optimized DNA extraction protocol obviated the need for WGA. WGA can be prone to allele dropout or other artifacts, but these were controlled for in the previous PAH study. Thus, an alternative explanation is that our results might represent a difference between *BMPR2* mutation-positive and mutation-negative cases. Yeager et al studied IPAH cases, and although their *BMPR2* status was not determined, mutations have been detected in fewer than half of IPAH patients.[6,7] In the present study, we focused on familial cases that were linked to *BMPR2*, including 3 with characterized mutations. MSI is apparently an uncommon phenomenon among this group of patients. Similarly, we examined whether somatic LOH at the *BMPR2* locus occurred in pulmonary vascular lesions from these patients. Heterozygosity was retained in all informative cases for 3 markers that encompass the *BMPR2* gene. These results suggest that somatic loss of the wild-type *BMPR2* allele in affected lung is not a function of disease progression

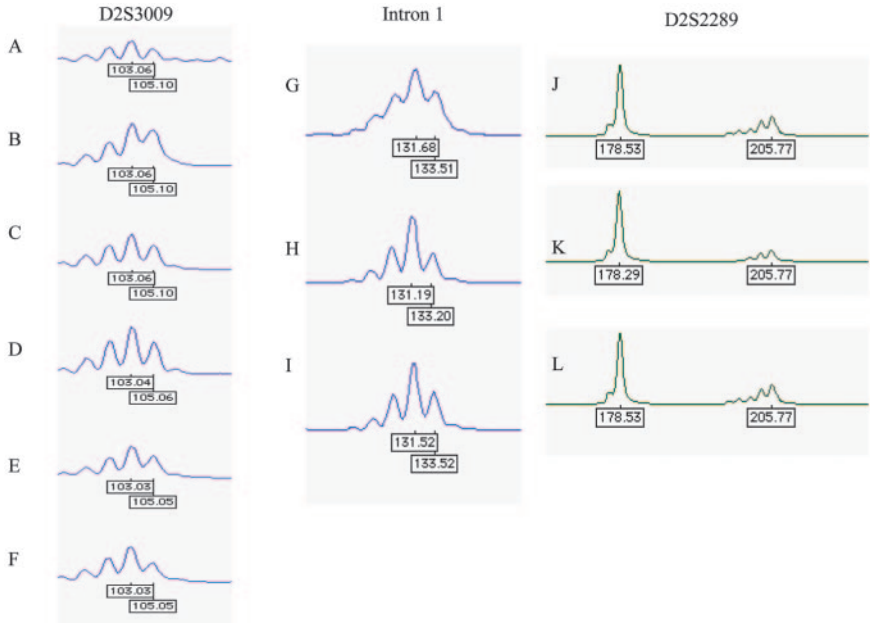

Figure 3. Heterozygosity at *BMPR2* locus is retained in FPAH lungs. Representative results are shown: D2S3009 in GER02, concentric (A and B) and plexiform (C, D, and E) lesions and normal lung (F); intron 1 in UK13, concentric (G) and plexiform lesions (H) and normal lung (I); and D2S2289 in UK14, concentric (J) and plexiform lesions (K) and normal lung (L).

in FPAH. However, we have previously noted an almost-complete absence of BMPR-II protein in FPAH lung on immunohistochemistry.[24] Thus other "second hits," such as somatic mutation, hypermethylation of the *BMPR2* promoter, or other factors secondary to perturbation of upstream regulatory proteins, remain possible mechanisms.

The *BMPR2* gene has been knocked out in the mouse by 2 independent groups in an effort to assess its importance in early development. As reported by Beppu et al,[25] although heterozygous mice harboring an ablated *BMPR2* allele appear phenotypically normal, mutant homozygotes die in utero at gastrulation. A second homozygous mutant of *BMPR2*, generated by the inframe deletion of exon 2, survived longer than the knockout model but was nevertheless lethal during embryogenesis.[26] These findings strongly argue that at least 1 functional *BMPR2* allele is necessary for survival, therefore raising the question of how the biallelic germline pathogenic mutation in the patient we describe herein was compatible with normal development and survival until adulthood.

TABLE 3. Summary of Results From MSI Analysis

Sample	BAT-25 (*c-Kit*)	BAT-26 (*MSH2*)	Bax	TGF-β2	BMPR2
GER02	P=0/3	P=0/3	P=0/3	P=0/2	P=0/2
	C=0/2	C=0/2	C=0/2	C=0/2	C=0/2
FRA01	P=0/5	P=0/5	P=0/5	P=0/5	P=0/5
	C=0/4	**C=1/3**	C=0/4	C=0/4	C=0/4
UK01	P=0/2	P=0/2	P=0/2	P=0/2	P=0/2
	C=0/2	C=0/2	C=0/2	C=0/2	C=0/2
UK07	P=0/2	P=0/2	P=0/2	P=0/1	P=0/2
	C=0/2	C=0/2	C=0/2	C=0/2	C=0/2
UK11	P=0/2	P=0/2	P=0/2	P=0/2	P=0/2
	C=0/2	C=0/2	C=0/2	C=0/2	C=0/2
UK13	P=0/1	P=0/1	P=0/1	P=0/1	P=0/1
	C=0/1	C=0/1	C=0/1	C=0/1	C=0/1
UK14	P=0/5	P=0/5	P=0/5	P=0/3	P=0/3
	C=0/3	C=0/3	C=0/3	C=0/2	C=0/2

All abbreviations are as defined in text or in the footnote to Table 2. Results are expressed as number of samples displaying MSI of the total number that were successfully amplified.

The R491Q mutation has been described in other PAH families[5] and is thus, clearly pathogenic. K512T has not previously been reported in PAH cases. However, this mutation was not detected among 150 control chromosomes. Furthermore, this mutation has previously been characterized functionally and was shown to perturb BMPR-II signaling.[11] It is therefore unlikely to be an innocuous polymorphism. A second possible explanation is that 1 of the 2 mutations arose postzygotically, and the patient might be a mosaic, with a sufficient proportion of wild-type alleles to rescue development. The patient's father was also affected with PAH, and thus, 1 mutation is presumed to be paternally inherited. Parental DNA samples were not available, and thus, it is not possible to determine whether the second mutation was inherited from the unaffected mother. However, no wild-type alleles were detected among 70 clones analyzed from each of blood and lung-derived DNA. This argues strongly against mosaicism resulting from a late-postzygotic mutation and suggests that patient 5559 is constitutionally compound heterozygous for 2 different *BMPR2* mutations.

Both of these missense mutations have been extensively characterized, and although both clearly perturb the BMPR-II signaling pathway, their effects are subtly distinct biochemically. The kinase $p38^{MAPK}$ is normally a downstream target of BMPR-II after activation of the receptor by ligand. However, both mutant receptors elevate the phosphorylation of this substrate constitutively, with the K512T mutation stimulating this effect to a significantly greater degree than R491Q. Perhaps the most critical distinction between these 2 mutations lies in their respective effects on activation of the Smad pathway. In luciferase reporter assays, the kinase domain mutation R491Q leads to a reduction of activation of the reporter to below endogenous levels, consistent with a

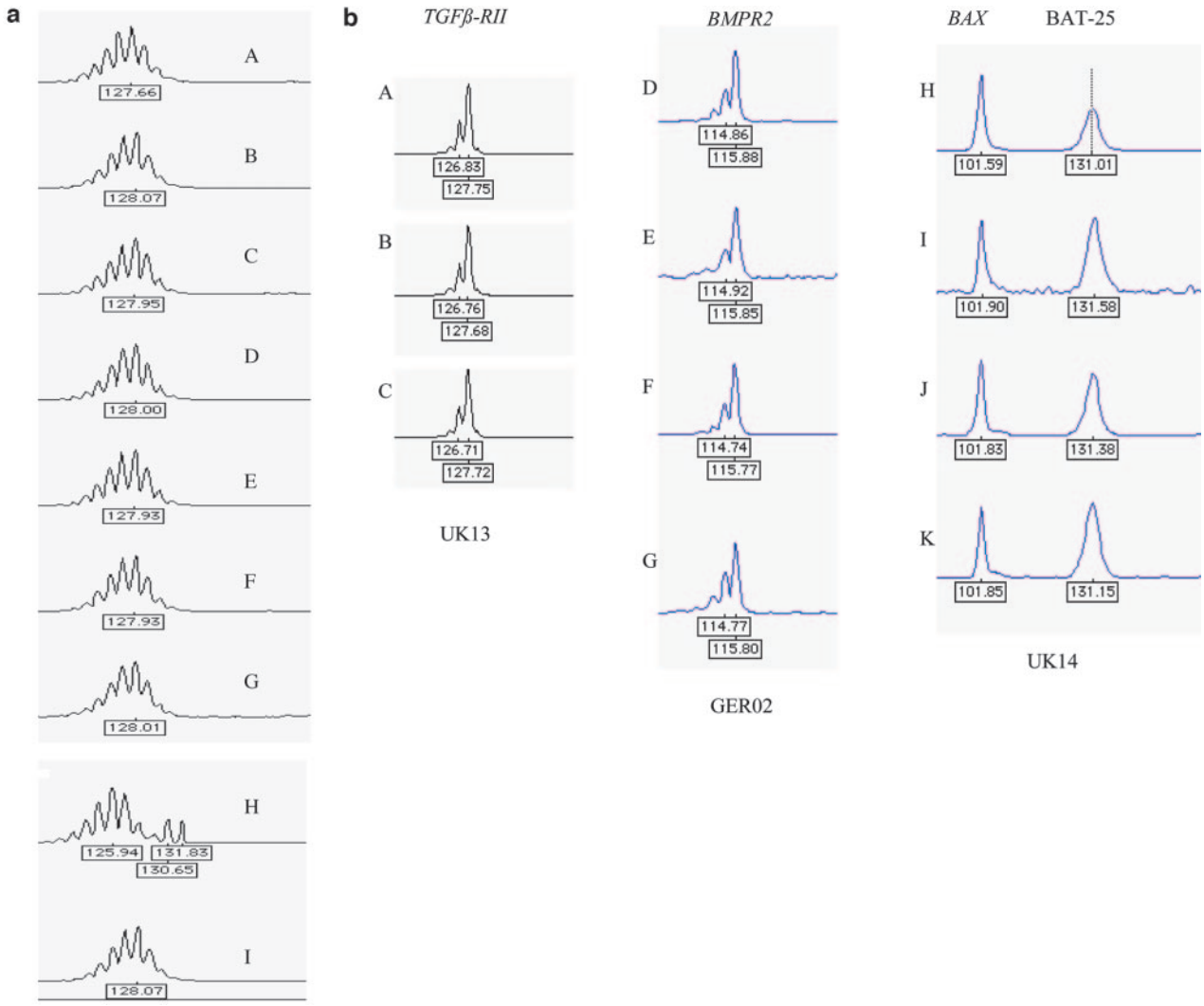

Figure 4. a, MSI detected at BAT-26 in single concentric lesion. Multiple abnormally sized fragments, indicative of MSI, are detected at BAT-26 in single concentric lesion (H) from case FRA01, compared with normal lung parenchyma (I). All other plexiform (A–E) and concentric (F and G) lesions were MSI-negative. b, Representative results for 4 other mononucleotide repeats in families of UK13, GER02, and UK14: concentric lesions (A, D, H, and I); plexiform lesions (B, E, F, and J); and normal lung parenchyma (C, G, and K).

dominant-negative effect on Smad signaling. In contrast, K512T reduced reporter gene activity by comparison with cells transfected with the wild-type receptor but did not compromise endogenous signaling activity.[11] We therefore propose that K512T is a pathogenic mutation that impairs BMPR-II signaling activity but retains sufficient residual Smad activity to permit normal embryonic development in the compound-heterozygote patient. The observation that despite apparently minimal BMPR-II activity, her age at PAH diagnosis and subsequent disease progression were comparable to those in individuals with heterozygous *BMPR2* mutations, and this suggests that further perturbation of BMPR-II signaling in heterozygous carriers is not a significant factor in the precipitation or progression of FPAH.

In conclusion, we have examined microdissected pulmonary vascular lesions from a series of FPAH patients. MSI was uncommon, being found in only 1 of 37 lesions for a single mononucleotide repeat. Furthermore, no evidence for LOH was found at the *BMPR2* locus. We have also investigated a patient with compound heterozygosity for 2 distinct *BMPR2* mutations. Surprisingly, the course of the disease in this patient was not dissimilar to the typical presentation and progression of PAH. Taken together, these 2 seemingly disparate sets of data suggest that somatic loss of wild-type *BMPR2* alleles or mutation of proapoptotic genes is unlikely to play a significant role in the pathogenesis of FPAH. The identification of other genetic or environmental factors that modulate the onset and progression of this disease remains an important priority.

Acknowledgments

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